Antigenic determinants of the bilobal cockroach allergen Bla g 2

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Abstract:

Bla g 2 is a major indoor cockroach allergen associated with the development of asthma. Antigenic determinants on Bla g 2 were analyzed by mutagenesis based on the structure of the allergen alone and in complex with monoclonal antibodies that interfere with IgE antibody binding. The structural analysis revealed mechanisms of allergen-antibody recognition through cation-π interactions. Single and multiple Bla g 2 mutants were expressed in *Pichia pastoris* and purified. The triple mutant K132A/K251A/F162Y showed an ~100-fold reduced capacity to bind IgE, while preserving the native molecular fold, as proven by x-ray crystallography. This mutant was still able to induce mast cell release. T-cell responses were assessed by analyzing Th1/Th2 cytokine production and the $CD4^+$ T-cell phenotype in peripheral blood mononuclear cell cultures. Although T-cell activating capacity was similar for the KKF mutant and Bla g 2 based on CD25 expression, the KKF mutant was a weaker inducer of the Th2 cytokine IL-13. Furthermore, this mutant induced IL-10 from a non-T-cell source at higher levels that those induced by Bla g 2. Our findings demonstrate that a rational design of site-directed mutagenesis was effective in producing a mutant with only 3 amino acid substitutions that maintained the same fold as wild type Bla g 2. These residues, which were involved in IgE antibody binding, endowed Bla g 2 with a T-cell modulatory capacity. The antigenic analysis of Bla g 2 will be useful for the subsequent development of recombinant allergen vaccines.

Keywords: allergen | aspartic protease | asthma | epitope mapping | immunotherapy | site-directed mutagenesis | x-ray crystallography | Bla g 2 | antigenic determinant | cockroach allergy

Article:

The abbreviations used are:
Bistris: 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol
PBMC: peripheral blood mononuclear cell
PDB: Protein Data Bank
CI: confidence interval.

Introduction

Sensitization to indoor allergens from mite, cockroach, cat, and dog is a risk factor for emergency room admissions with asthma. Since the original reports by Bernton and Brown (1), the importance of cockroach allergy for the development of asthma has been confirmed by multiple studies, especially in inner cities among lower socio-economic groups (2,–4). Inner City Asthma Studies in the United States established that cockroach allergy affects up to 81% of asthmatic children that are sensitized and exposed to cockroach allergens (5, 6). In Taiwan, 58% of asthmatic patients are allergic to cockroaches (7). Most allergy treatments are aimed at reducing symptoms, and only allergen immunotherapy using natural allergen extracts, which is currently used in the United States and other countries, can modify the course of the disease. This effect has been attributed to down-regulation of Th2-driven allergic inflammatory responses. However, specific immunotherapy is not frequently used for cockroach allergy. Recent pilot studies showed that immunotherapy for cockroach allergy is more likely to be effective when administered subcutaneously (8). Large scale randomized and controlled clinical trials with a longer follow up that use well characterized cockroach extracts and/or recombinant alternative allergens are required to establish efficacy and safety of cockroach immunotherapy (9).

With the advent of molecular biology, nine allergens from *Blattella germanica* and nine from *Periplaneta americana* have been cloned in the last 15 years and are listed in the official database of allergen nomenclature that is approved by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Subcommittee. Natural or recombinant cockroach allergens have been used to evaluate their importance in allergic disease. Of all *B. germanica*allergens, patients show the highest prevalence of sensitization (∼55–70%) to Bla g 2, and this major allergen was therefore selected as the main focus of our studies (10, 11). A Bla g 2 homolog from *P. americana* also showed a high prevalence of sensitization (81%) among patients with persistent asthma and rhinitis in Taiwan (7). Bla g 2 has a typical bilobal fold of aspartic proteases (*i.e.* pepsin, renin, and chymosin), but distortions in the area corresponding to the active site, due to amino acid substitutions in the canonical catalytic triads, make Bla g 2 devoid of the standard protease activity (12). Bla g 2 is considered quasi-symmetrical because both lobes, although not identical in amino acid sequence, are structurally equivalent to two identical subunits that are present in the related HIV aspartic proteases, defining their typical 2-fold symmetry (13).

The goal of this study was to analyze the antigenic surface of Bla g 2 to gain insight into allergen-antibody interactions and to obtain information that can be used in the future for the design of engineered recombinant hypoallergen mutants with the capacity to induce T-cell reactivity. The primary objective was to mutate residues involved in different IgE antibody-

binding sites that had been either previously identified by mutagenesis of two Bla g 2 epitopes defined by x-ray crystallography or newly identified in this study (13,–16). Our findings show mechanisms of allergen-antibody interaction and reveal that Bla g 2 mutants displaying the same overall fold as the wild type allergen display reduced capacity to bind IgE but retain the ability to activate T-cells and modulate their response.

Experimental Procedures

Human Subjects. Serum samples (n = 44) from cockroach-allergic patients for *in vitro* analysis of IgE reactivity were obtained from three different sources as follows: 1) a commercial source (Bioreclamation, Inc., Westbury, NY); 2) leftover deidentified samples collected from patients enrolled in 1988–1989 in Wilmington, DE, and Charlottesville, VA, for epidemiological studies performed at the University of Virginia (2, 17); and 3) kindly provided by Dr. Robert Wood, from The Johns Hopkins University, Baltimore, MD, as part of a collaborative study with the Inner City Asthma Consortium (18). Bioreclamation operates in full compliance with the Food and Drug Administration guidelines. Three sera from Bioreclamation had anti-cockroach IgE levels of 284 kilounits/liter (range 16–811 kilounits/liter) by ImmunoCAP (Phadia AB, Uppsala, Sweden). The studies performed at The Johns Hopkins University were approved by the Human Investigation Committee, and blood was drawn after informed written consent was obtained from the patient. IgE antibody levels in sera ranged from 25 to 640 ng of total IgE/ml (average 1721 ± 1722 ng/ml) (2), and 6–164 ng of IgE against Bla g 2/ml (average 56 ± 45 ng/ml), measured by mAb-based RIA (10). A pool was prepared by mixing sera from six cockroach allergic patients (total IgE, 42 ng/ml; range 25–219 ng/ml; Bla g 2-specific IgE of 33 ng/ml; range 45–164 ng/ml). Finally, sera were selected out of 31 patients recruited at The Johns Hopkins University, under the NIAID Protocol Number ICAC-18 (18). Patients from the Baltimore area had a history of allergic rhinitis, asthma, and sensitivity to cockroach. Two sera selected due to their highest Bla g 2 antibody titers had total IgE levels of 2,152 and 1,852 kilounits/liter, cockroach-specific IgE greater than 100 kilounits/liter, and Bla g 2-specific IgE of 12.5 and 9.03 kilounits/liter, respectively.

TABLE 1. Human subjects for T cell studies. CRE is *B. germanica* extract.

	Subject	CRE (i6)	Bla g 2	Total IgE
Cockroach-allergic	1	0.59 (class 1)	3.16 (class 2)	2464
	2	6.64 (class 3)	15.9 (class 3)	1849
	3	1.56 (class 2)	0.35 (class 1)	6878
	4	2.26 (class 2)	<0.35 (negative)	374
	5	9.57 (class 3)	<0.35 (negative)	374
	6	1.87 (class 2)	<0.35 (negative)	132
Controls	7	<0.35 (negative)	<0.35 (negative)	3.4
	8	<0.35 (negative)	<0.35 (negative)	26.98
	9	<0.35 (negative)	<0.35 (negative)	142
	10	<0.35 (negative)	<0.35 (negative)	304

Cockroach allergic and non-allergic subjects (age >18 years) were recruited through the University of Virginia Allergy Division to evaluate whether Bla g 2 mutants retained T-cell reactivity (Table 1). Cockroach-allergic subjects were defined as those with IgE antibody titers to *B. germanica* (i6 ≥ class I) with or without IgE antibodies to Bla g 2 measured by Phadia ImmunoCAP assay (Uppsala, Sweden) and streptavidin ImmunoCAP assay, respectively (19).

Non-allergic subjects had no measurable serum IgE antibodies to cockroach extract or Bla g 2. T-cell studies were approved by the University of Virginia Human Investigations Committee (protocol 13166).

Site-directed Mutagenesis of Bla g 2. Mutations were sequentially performed on a template Bla g 2 molecule (recombinant Bla g 2-N93Q, with intact epitopes for mAb 7C11 and 4C3), which is the allergen that was originally crystallized and used as reference Bla g 2 in the experiments performed in this study (13). The N93Q substitution was designed to prevent glycosylation of Bla g 2 that interferes with crystallization and is distant from the epitopes for mAb 7C11 and 4C3. The Bla g 2-DNA was inserted into the yeast *Pichia pastoris* expression vector pGAPZαC for constitutive expression of the allergen. Site-directed mutagenesis was performed using QuikChange™ (Stratagene La Jolla, CA). Sequence of the mutated DNA was confirmed before linearization and transformation into the *P. pastoris* strain KM71.

Expression, Purification, Quantification, and Folding Analysis of Bla g 2 Mutants. Expression of recombinant proteins in *P. pastoris* was performed as described (12, 16). Reference Bla g 2 and mutants were purified by affinity chromatography using either mAb 7C11, 4C3, or 2F1, depending on the location of the mutation. Purity and integrity of the proteins were assessed by SDS-PAGE, and protein content was measured by Advance Protein Assay (Cytoskeleton, Denver, CO). Overall folding of the mutants was assessed by CD spectroscopy or x-ray crystallography, complemented with confirmation of antibody binding by performing dose-response ELISA. For this, the mAb 2F1 was used as capture antibody and a Bla g 2-specific rabbit polyclonal antibody (pAb) for detection (at 1:50,000 dilution) (n = 3 assays). A standard containing natural Bla g 2 was used starting at 250 ng/ml. Peroxidase-labeled goat anti-rabbit IgG was added, the plates were incubated for 1 h followed by the addition of 2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt/H_2O_2 as a substrate, and absorbance was read at 405 nm using an absorbance microplate reader (BioTek Instruments, Inc., Winooski, VT).

Analysis of recombinant mutants by circular dichroism (CD) and mass spectrometry was performed as follows. The far-UV CD spectra were recorded at concentrations around 0.1 mg/ml upon addition of 10 mmsodium phosphate, pH 7.4, between 190 and 260 nm, using a Jasco J-810 spectropolarimeter (Japan Spectroscopic), temperature-controlled at 20 °C. NanoLC-MS/MS-based peptide mapping (CapLC, Micromass-Waters) of proteolytic digests was performed using trypsin and V8 protease (Roche Applied Science). MS/MS data were evaluated automatically using PLGS 2.2.5TM software. An in-house allergen sequence data bank was used for MS/MS data search in nonspecific digestion mode with carbamidomethylation set as fixed modification. The structure of the triple mutant KKF was solved by x-ray crystallography as described below.

X-ray Crystallography of the Triple Bla g 2 Mutant KKF. The original sample of the KKF mutant of Bla g 2 was in PBS buffer, which was exchanged for 20 mm Tris, pH 7.5, with added 0.1 m NaCl. Before crystallization, the sample was concentrated to 6 mg/ml, and crystals were grown in Linbro plates using the hanging-drop vapor diffusion method. Crystals used for data collection were obtained in drops containing 4 μl of sample and 2 μl of well solution, the latter containing 20% PEG 8K, 0.2 m magnesium acetate, and 10 mm DTT in 0.1 m Bistris[3] buffer, pH 6.5. X-ray diffraction data extending to 2.9 Å were collected at the SER-CAT ID-22 beamline at the Argonne Advanced Photon Source, using a MAR 300 CCD detector. The crystals of the

mutant were not isomorphous to the crystals of the apo-Bla g 2 and contained four molecules in the asymmetric unit. The structure was solved by molecular replacement using the program PHASER (20), with the coordinates of the 1.3 Å structure of apo-Bla g 2 (PDB code 1YG9) used as the initial search model. The solution was rebuilt with COOT (21) and refined with REFMAC5 (22). Figures were prepared with PyMOL (23).

IgE Reactivity of rBla g 2 Mutants. Direct IgE antibody binding to reference Bla g 2 and mutants with substitutions in new potential IgE antibody-binding sites was performed by multiplex fluorescent array. Monoclonal antibodies 7C11 (20 mg) were coupled to Luminex carboxylated fluorescent microsphere bead sets (Luminex Corp., Austin, TX), and the multiplex fluorescent array was performed as described (24, 25). The mAb coupled beads were added along with rBla g 2-N93Q or mutants at a concentration of 400 ng/ml. The rBla g 2-N93Q and the mutants F162Y, F300Y, mutant A, and mutant B were tested using beads coated with the mAb 7C11. Sera (ranging from 1:2 to 1:20 dilution) or anti-Bla g 2 rabbit pAb (1:1,000 dilution) were added to wells, mixed with beads, and incubated. Biotinylated mAbs 4C3 or 2F1 (at 1:1,000 and 1:20,000, respectively) were added to the wells where a mAb sandwich assay was performed. Detection of human IgE or rabbit IgG and final assay development and measurement of absorbance were performed as described previously (16). Absorbances for each mutant were compared with rBla g 2-N93Q by paired, Student's *t* test. *p* values lower than 0.001 were considered significant.

Inhibition ELISAs were performed to compare the capacity for Bla g 2 mutants to inhibit IgE antibody binding to Bla g 2. In brief, Bla g 2 and mutants were pre-incubated in tubes for 1 h at room temperature with IgE antibodies (sera pool or individual sera were diluted from 1:4 to 1:2) and then added to microtiter plates coated with 10 μg/ml rBla g 2-N93Q. After 3 h of incubation, plates were washed and incubated for 1 h with peroxidase-labeled goat anti-human IgE and developed as for quantification by ELISA as described above.

Mediator Release Assays Using Human Mast Cell Culture. Normal human skin tissue was received from the Cooperative Human Tissue Network, with approval by its Human Studies Institutional Review Board. Fresh samples of skin tissue were cut into 1–2-mm fragments and incubated multiple times in a solution of tissue-digesting enzymes, as described previously (26). After purification, mast cells were sensitized overnight with allergen-specific IgE-containing serum (1:10–1:100 dilutions depending on IgE titer). Optimal serum concentrations eliciting maximal release were determined before testing the mutants, by sensitizing mast cells overnight with varying concentrations (dilutions 1:10 to 1:100) of four different sera (F1, G1, K2, and L2), and activating them with varying concentrations of rBla g 2 (data not shown). After determining optimal sera and concentrations (F1 and G1; 1:10), cells were washed and challenged with varying concentrations of rBla g 2, mutants or positive controls for 20 min. β-Hexosaminidase release was measured in the supernatants obtained as described previously (26, 27). Experiments included positive controls (anti-FcεRI antibodies and wild type antigens), untreated cells (spontaneous), and increasing doses of rBla g 2 mutants (N93Q, Lys-132, Lys-251, F162Y, KK, and KKF; 0.001–20 μg/ml). Wild type antigens included natural Bla g 2 (nBla g 2), recombinant Bla g 2 (rBla g 2), and German cockroach extracts (Greer, Lenoir, NC). All samples were analyzed in duplicate from at least three separate donors to determine total β-hexosaminidase

release. Significance was assessed using Student's *t* test. *p* values lower than 0.05 were considered significant.

T-cell Epitope Prediction. Predictions of 9-mer peptide core epitopes of wild type and KKF mutant protein sequences were performed for 406 HLA-DRB1 alleles using MULTIPRED2 (28). These alleles belong to 13 HLA-DR supertypes (DR1, DR3, DR4, DR6, DR7, DR8, DR9, DR11, DR12, DR13, DR14, DR15, and DR16) and provide close to 100% coverage of the general population. To account for contributions of flanking residues to MHC binding, 15-mer epitope predictions were performed for the following major HLA-DRB1 alleles: 0101, 0301, 0401, 0701, 0801, 0901, 1101, 1201, 1301, 1401, 1501, and 1601 using NetMHCIIpan (version 3.1) (29). Binding predictions for the 0601 allele were not available using this tool. For both methods, predicted binding affinities with an IC_{50} of $\leq$500 nm were considered to be weak binders, whereas predicted binding affinities of IC_{50} of $\leq$50 nm were considered strong binders.

Peripheral Blood Mononuclear Cell Cultures. Freshly isolated peripheral blood mononuclear cells (PBMCs) were stimulated with *B. germanica* extract or recombinant allergens (10 μg/ml) in 24-well plates (1×10^6cells/ml) using established methods (30). On day 7, cells were re-stimulated with phorbol 12-myristate 13-acetate (50 ng/ml; Fisher) and 2 μg/ml ionomycin (Invitrogen) in the presence of brefeldin A (BD Biosciences) for 4 h. Cells were then stained for surface and intracellular markers and analyzed using an LSRII flow cytometer (BD Biosciences). Monoclonal antibodies used for surface staining in flow cytometric studies were as follows: Alexa Fluor 488 anti-IL-10 (clone JES3-9D7; Biolegend, San Diego); V450 anti-IFN-γ (B27; BD Biosciences); phycoerythrin anti-IL-4 (MP4-25D2; Biolegend); phycoerythrin-Cy7 anti-CD3 (SK7; BD Biosciences); (*R*)-phycoerythrin-Texas Red anti-CD8 (3B5; Invitrogen); and allophycocyanin-Cy7 anti-CD25 (M-A251; BD Biosciences). Live cells were identified using Fixable Aqua Dead Cell Stain (Invitrogen). Data were acquired using FACS Diva software (version 6.0, BD Biosciences). Because CD4 is down-regulated on T-cells after activation with phorbol 12-myristate 13-acetate and ionomycin (31, 32), live $CD3^+CD8^-$ cells were analyzed to capture total $CD4^+$ T-cell events, after excluding monocytes and B-cells. Culture supernatants were assayed for cytokines by cytometric bead assay (Millipore, Billerica, MA) using a Bioplex System (Bio-Rad).

Statistical Analysis of T Cell Data. T-cell frequencies and secreted cytokines induced by different stimuli were analyzed within groups and between groups after logarithmic transformation using linear mixed models and pairwise comparisons. Antilog transformations were used to construct 95% confidence intervals for geometric mean values and to compare the ratio of geometric means. Adjusted *p* values were generated using Tukey's 0.05 type I error rate or the Bonferroni 0.05 type I error rate where appropriate.

Results

Rational Design of Site-directed Mutagenesis. The strategies for mutagenesis were based on the crystal structures of Bla g 2 alone and in complex with the fragments of the mAb 7C11 and 4C3 (PDB codes 1yg9, 2nr6, and 3liz, respectively). Mutants of Bla g 2 were generated based on prior knowledge of antibody-binding sites and structural considerations using the following three strategies. 1) To combine mutations of two lysine residues involved in IgE binding to Bla g 2

(so-called double mutant containing K132A and K251A). These lysine residues are located in two discrete conformational epitopes that were previously identified by x-ray crystallography and site-directed mutagenesis studies of Bla g 2 complexed with monoclonal antibodies 7C11 and 4C3 (Fig. 1, *a–c*) (14,–16). 2) To investigate whether phenylalanines (Phe-162 and Phe-300) contribute to IgE binding, because of the reported involvement of phenylalanines in human IgE and IgG antibody binding and the enrichment of this residue within Bla g 2 (21 of 328 residues) (Fig. 1, *a, d,* and *e*) (33,–35). Single mutants F162Y and F300Y were expressed. 3) To examine whether the quasi-symmetry of the bilobal Bla g 2 molecule would inform the location of a heretofore unidentified IgE antibody-binding site located on the opposite lobe of the molecule to the known epitope for mAb 7C11 located on the N-terminal lobe. The C-terminal lobe was mutated to create mutant A E240A/R242cA/K246A and mutant B K241A/R243A (see design below) (Fig. 1, *a, f,* and *g*). The second and third strategies for mutagenesis aimed to investigate additional potential IgE antibody-binding sites. Finally, a triple mutant was expressed by combining mutations in three different epitopes, which impair IgE antibody binding. The triple mutant was K132A/K251A/F162Y (designated KKF). The terms single, double, and triple mutant refer to the number of mutations in addition to the N93Q present in the reference construct. Names assigned to all the Bla g 2 mutants are shown in Table 2.

Design of the Bla g 2 Mutants. For the first and main approach for mutagenesis, lysines 132 and 251 were mutated (to alanine) given their proven important role in recognition of IgE and IgG antibodies (14,–16). Both lysines were able to form cation-π interactions with tyrosines from mAb 7C11 and mAb 4C3, respectively, as well as hydrogen bonds with aspartates from these antibodies. The interactions of these lysines with the respective antibodies were formed in a very similar structural context (Fig. 1, *b* and *c*) (14,–16).

The second strategy for mutagenesis aimed to investigate whether phenylalanines would be involved in Bla g 2 interactions with IgE antibodies. The strategy was based on the fact that Bla g 2 contains a large number of phenylalanines, and this residue is to be involved in allergen-antibody interactions (33,–35). Aromatic residues such as phenylalanine might also favor peptide binding to MHC molecules and consequent T-dependent antibody responses (34). Of the 21 phenylalanines in Bla g 2, only four (5, 158, 162, and 300) showed a favorable position and orientation on the allergen surface for potential antibody binding. Two of these were selected and mutated to tyrosines as follows: 1) Phe-162 because it was located in the middle of the cluster of aromatic residues that include two other phenylalanines, Phe-5 and Phe-158, all of which could potentially contribute to antibody binding, through either a cation-π interaction or at least one hydrogen bond (Fig. 1*d*); and 2) Phe-300 that is surrounded by several charged residues comprising Lys-218, His-298, and two aspartates (Asp-76 and Asp-215) (Fig. 1*e*). Phe-162 differs from Phe-300 regarding the structural context in Bla g 2, and both residues make a different set of contacts with surrounding residues: Phe-162 does not form cation-π interactions, but it forms hydrophobic stacking interactions with two phenylalanines (5 and 158), as well as a weak ionic interaction with Glu-160 (the phenylalanine ring has a negative partial charge above the plane of the ring, utilized in cation-π interactions, and a partial positive charge around the perimeter of the ring) (Fig. 1*d*). Phe-300 seems to form cation-π interactions with His-298, hydrophobic interactions with Lys-218, and weak ionic interactions with two aspartates, 76 and 215, in a manner reminiscent of the interactions between Phe-162 and Glu-160 described above (Fig. 1*e*). Phe-162 and Phe-300 were mutated to tyrosine (which only differs from phenylalanine

by addition of an extra hydroxyl) to maintain the structural environment in the vicinity of the aromatic ring, by preserving its interactions with the surrounding residues (Fig. 1, *d* and *e*).

A third strategy for mutagenesis was based on utilization of symmetry. This new approach for epitope prediction was experimentally investigated due to the quasi-symmetrical nature of the bilobal molecule of Bla g 2. This approach embraces a few of the criteria used by bioinformatics tools for prediction of B-cell epitopes (surface accessibility, protruding areas, or geometrical shape). The aim was to test whether the molecular pseudo-symmetry of Bla g 2 would allow mapping the presence of an additional IgE antibody-binding epitope opposite the one present on the N-terminal lobe of Bla g 2. That epitope would include the residues that are in equivalent positions to residues of the mAb 7C11 epitope, but they would not be expected to be responsible for antibody cross-reactivity given their different nature. The selection of residues to mutate was based on the fact that Bla g 2 shares the same structural template that is unique to aspartic proteases that include the HIV proteases and pepsin-like proteases. The retroviral aspartic proteases, exemplified by the enzyme from HIV, are composed of two identical subunits that assemble to form a dimeric enzyme with a 2-fold symmetry. In contrast, pepsin-like aspartic proteases, including Bla g 2, have a bilobal shape due to an evolutionary process that involved duplication, fusion, and mutagenesis of an original subunit. This process resulted in the formation of a bilobal protein, considered pseudo-symmetrical, with two lobes of low amino acid identity (~15% for Bla g 2) (36). The identical nature of the two monomers from a dimeric HIV was taken into consideration to identify the equivalent location of residues opposite the mAb 7C11 epitope, which overlaps with an IgE-binding site, in the N-terminal lobe. Bla g 2 and the human HIV protease (PDB code 5HVP) were superimposed in the same orientation (Fig. 1, *f* and *g*). Such comparisons allowed marking of the residues occupying structurally equivalent positions in both lobes of Bla g 2. There was no residue in the C-terminal domain in an equivalent position to the lysine 132 that was important for IgE antibody binding. Therefore, several other residues from the mAb 7C11 epitope in the N-terminal domain were chosen to provide reference residues for the search of their possible counterparts in the C-domain of Bla g 2: Lys-65, Asp-68a ("a" indicates that Asp-68 is an amino acid insertion *versus* pepsin, used as reference for numbering the sequence), Arg-83, and Glu-86. Two mutants were designed to modify the residues in the C-terminal lobe of Bla g 2 that are spatially equivalent to the residues of mAb 7C11 epitope in the N terminus: (*a*) mutant A, with substitutions E240A and K246A, that are related by pseudo-symmetry to Lys-65 and Glu-86, respectively, from the mAb 7C11 epitope, as well as R242cA ("c" indicates also an insertion *versus* pepsin), that is located very close to them and with similar orientation (Fig. 1*f*). In HIV protease, residues Arg-41 and Gln-61 are equivalent in position to Lys-65 and Glu-86 from Bla g 2, respectively. (*b*) Mutant B with K241A and R243A (in similar positions to Asp-68a and Arg-83 from the mAb 7C11 epitope) (Fig. 1*g*). In HIV protease, Arg-57 is equivalent to Arg-83 in Bla g 2, which is equivalent by symmetry to Arg-243 in the C-terminal lobe. In HIV there is not a residue in an equivalent symmetrical position to Asp-68a. Therefore, Lys-241 was selected because of its proximity and orientation toward Arg-243 in the C-terminal lobe. The disposition of Lys-241 and Arg-243 closely resembles the mutual disposition of the residues Asp-68a and Arg-83, respectively, in the mAb 7C11 epitope. Most mutations involved replacement of residues by alanine, as a way to evaluate the involvement of their side chains in the interaction with the antibody.

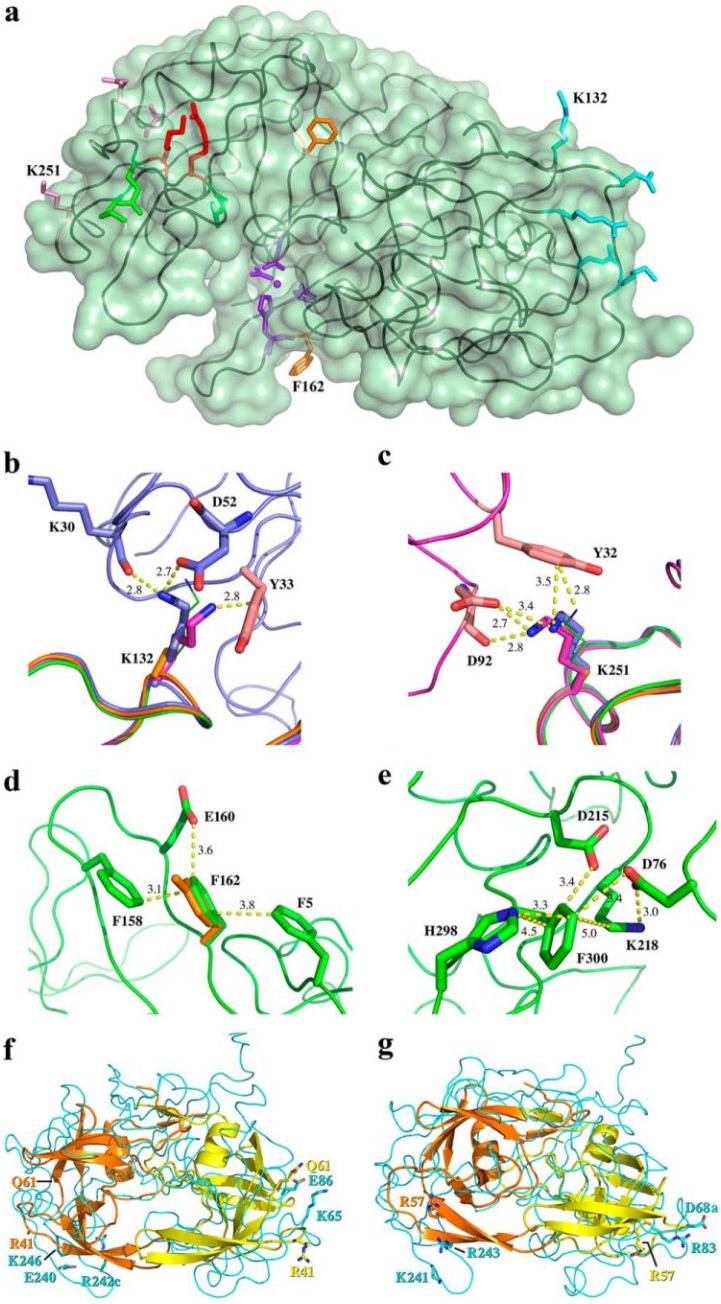

FIGURE 1. Rational design of site-directed mutagenesis. *a,* overview of residues involved in mAb-binding epitopes and potential IgE antibody-binding sites. Residues that form epitopes in Bla g 2 interacting with mAb 7C11 (*cyan,* including Lys-132) and mAb 4C3 (*pink,* including Lys-251), as well as side chains of Phe-162 and Phe-300 (*orange*), and substitutions in mutants A (*green*) and B (*red*) are shown as *sticks*. Residues comprising the zinc-binding site (His-155, His-161, Asp-303, and Asp-307) are shown as *purple sticks. b* and *c,* importance of lysines for antibody recognition. Comparison of the relative positions of each lysine (132 in *b* and 251 in *c*) in Bla g 2 before (*green,* PDB code 1YG9) and after (*purple,* PDB code 2NR6, allergen bound to mAb 7C11, and *magenta*, PDB code 3LIZ, allergen bound to mAb 4C3) binding to the respective mAb 7C11 (*purple*) or 4C3 (*magenta-pink*). The corresponding fragments in the KKF mutant are shown in *orange. d* and *e,* two phenylalanines (Phe-162 in *d* and Phe-300 in *e*, shown in *green*) were selected as potential IgE antibody-binding sites from 21 phenylalanines present in Bla g 2; orientation of the side chain of F162Y in the KKF mutant (*d*) is shown in *orange. f* and *g,* mutants A (*f*) and B (*g*) were designed by superimposing HIV protease (subunits *orange* and *yellow,* ribbon representation) on Bla g 2 (*cyan*). The residues in the C-terminal lobe of Bla g 2 (*left*) are identified through pseudosymmetry to the residues in the N-terminal lobe within the epitope for mAb 7C11 (*right*).

TABLE 2. Bla g 2 mutants and strategies for site-directed mutagenesis

Mutant name	Substitutions	SM[a]	Purpose of the mutation
Double mutant (KK)	K132A/K251A	1	Epitopes for mAb 7C11 and 4C3 that interfere with IgE antibody binding
Triple mutant (KKF)	K132A/K251A/F162Y	1, 2	Epitopes for mAb 7C11 and 4C3 and explore Phe on molecular surface
F162Y	F162Y	2	Explore Phe on molecular surface
F300Y	F300Y	2	Explore Phe on molecular surface
Mutant A	E240A/R242cA/K246A	3	Explore Bla g 2 symmetry by mutating site located at the opposite lobe from mAb 7C11
Mutant B	K241A/R243A	3	Explore Bla g 2 symmetry by mutating site located at the opposite lobe from mAb 7C11

[a] SM indicates strategies for mutagenesis.

Structural Comparisons Reveal Proper Folding of the KKF Mutant and a Mechanism of Allergen-Antibody Interaction Involving Lysines. The crystal structure of the KKF triple mutant, determined at a resolution of 2.9 Å, showed that the three substitutions did not affect the overall fold of Bla g 2 (Fig. 2). The statistics of data collection and refinement are listed in Table 3. Overlap of the structures of the triple mutant and the reference molecule rBla g 2-N93Q confirmed the intended amino acid substitutions and showed a root mean square deviation of Cα positions of 0.009 Å (Fig. 2).

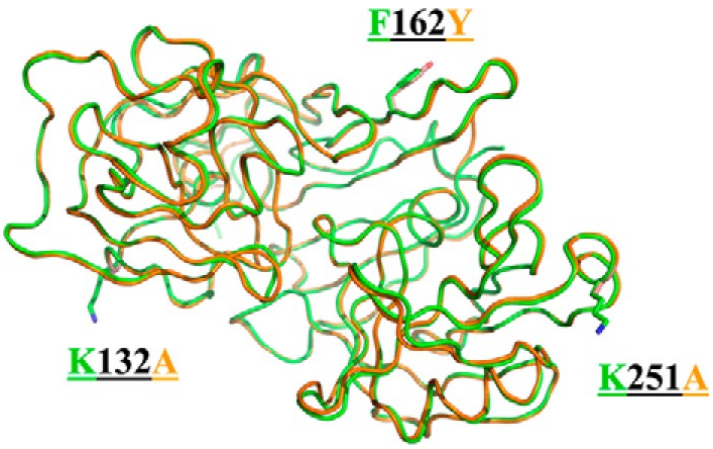

FIGURE 2. X-ray crystal structure of the triple mutant. X-ray crystal structure of the reference molecule rBla g 2-N93Q (*green*) superimposed on the triple mutant K132A/K251A/F162Y (*orange*) showing the same overall folding of both molecules.

A comparison of the relative positions of lysines 132 and 251 in overlapped structures of Bla g 2 alone (Bla g 2-N93Q and KKF) and in complex with the mAb 7C11 and 4C3 shows that the lysines undergo a conformational change upon antibody binding. In the first stage, their orientation allows them to form a cation-π interaction with a tyrosine residue in the antibody within the epitopes (H1/Lys-33 with Lys-132 or L1/Tyr-32 with Lys-251). Subsequently, both lysine side chains slightly changed their conformation to form stronger ion pairs with the aspartates located nearby (H2/Asp-52 with Lys-132 and L3/Asp-92 with Lys-251) (Fig. 1, *b* and *c*). This change allows the formation of either a cation-π interaction for initial antibody recognition or ion pairs for subsequent strong antibody binding (Fig. 1, *b* and *c*).

TABLE 3. Crystallography data for the triple mutant KKF

Wavelength (Å)	1.000
Space group	$P2_12_12_1$
Unit cell parameters (Å)	a = 66.8, b = 75.4, c = 339.9
Resolution (Å)	50.0 to 2.9
No. of reflections (unique/total)	34,440 (184,929)
Completeness (%) (last shell)	88.5 (45.3)
R_{merge} (%)	8.6 (29.8)
No. of molecules in a.u.	4
No. of protein atoms	10,252
No. of solvent molecules	40
No. of heteroatoms	32
R_{cryst}	19.6%
R_{free} (10% of data)	25.7%
Root mean square deviations from ideality	
Bond lengths	0.009 Å
Bond angles	1.18°

The CD spectra of mutants A and B, natural Bla g 2, and the rBla g 2-N93Q reference molecule were equivalent, with minima between 205 and 215 nm and maxima below 200 nm, indicating a similar overall folding of the four molecules (Fig. 3). The mutations were confirmed by nanoLC-MS/MS-based protein sequencing (data not shown). Antibody-binding experiments showed parallel ELISA dose-response curves for rBla g 2-N93Q and rBla g 2 mutants (data not shown), confirming that the conformational epitopes for mAb, dependent on an integral structure, were preserved. The substitutions in the KKF mutant did not affect the zinc-binding site in Bla g 2 (involving His-155, His-161, Asp-303, and Asp-307) (Fig. 1*a*). Substitutions in mutants A and B were not expected to affect the zinc-binding site due to their distant position in the molecule (Fig. 1*a*).

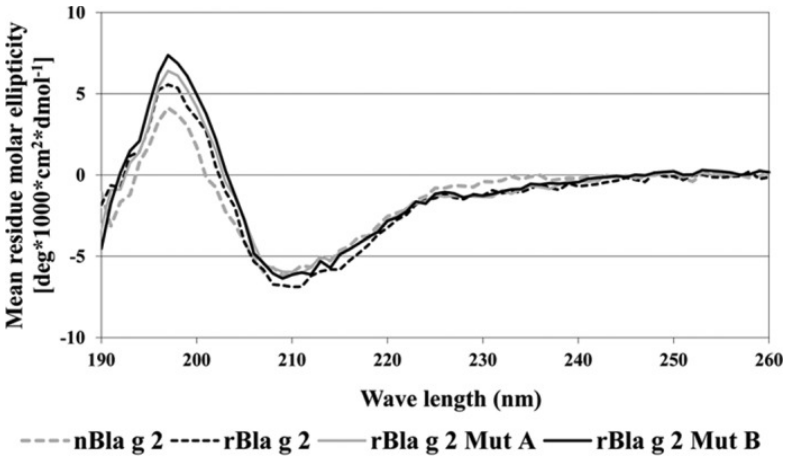

FIGURE 3. CD spectra of Bla g 2 and mutants of site opposite the mAb 7C11 epitope (mutant A (*Mut A*) and mutant B (*Mut B*)).

Analysis of IgE Antibody Binding Identified Phe-162 as a Key Residue Involved in IgE Recognition. To identify new IgE antibody-binding sites on Bla g 2, the mutants F162Y, F300Y, mutant A, and mutant B were screened for reduction of direct IgE antibody recognition using 10 sera from cockroach-allergic patients, as described previously (16). Mutants were presented in a

defined orientation by using mAb 7C11 as capture antibody. Therefore, the corresponding epitope for mAb 7C11 (intact in these mutants) would be excluded in the captured molecule from binding IgE, which would facilitate the detection of the effect of mutations on IgE antibody binding to the rest of the molecule. Among the four mutants tested, only F162Y showed a reduced capacity to bind IgE (Fig. 4*a*). Therefore, mutation F162Y was added to the KK double mutant for the expression of the KKF triple mutant. The binding by the control antibodies (anti-Bla g 2 pAb, mAb 2F1, and mAb 4C3) was similar for the four mutants, as expected for correctly folded molecules (Fig. 4*b*). In confirmation of the importance of these three mutations for IgE antibody binding, KKF showed more than 10 times reduced IgE antibody binding than the multiple mutants A or B in a highly cockroach-allergic patient by inhibition assay (data not shown).

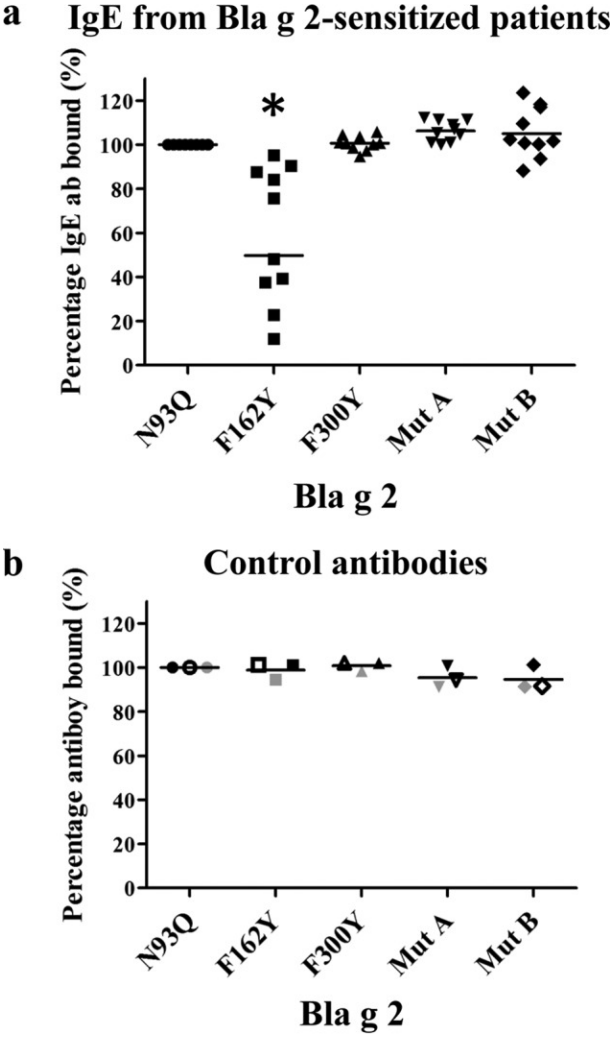

FIGURE 4. IgE antibody bound to Bla g 2 reference and single mutants using multiplex array. Recognition of Bla g 2 mutants (presented by mAb 7C11 coupled to beads) by IgE antibodies from Bla g 2-sensitized cockroach allergic patients (n = 10) (*a*). *b,* control Bla g 2-specific antibodies: pA (*black-filled symbols*), mAb 2F1 (*black open symbols*), and mAb 4C3 (*gray-filled symbols*). Scatter plots show geometric mean of percentages of antibody bound to each mutant *versus* rBla g 2-N93Q for each data set. The *asterisk* shows significant difference of the absorbances for a mutant *versus* rBla g 2-N93Q ($p < 0.001$). Mutant A (*Mut A*) and mutant B (*Mut B*).

Single (K132A, K251A, or F162Y), double (KK), and triple (KKF) mutants were compared by IgE inhibition assays. Double and triple mutants showed the most reduced capacity to inhibit IgE antibody binding *versus* the reference Bla g 2. The reduction of IgE antibody binding varied by patient and mutant. The strongest effects (~100-fold) were found for mutants KK and KKF (Fig. 5). The mutant KKF was selected for functional assays because it has substitutions in a larger number of epitopes (three *versus* two in KK), and therefore it has the potential to lead to a stronger reduction in IgE antibody binding and T-cell effects.

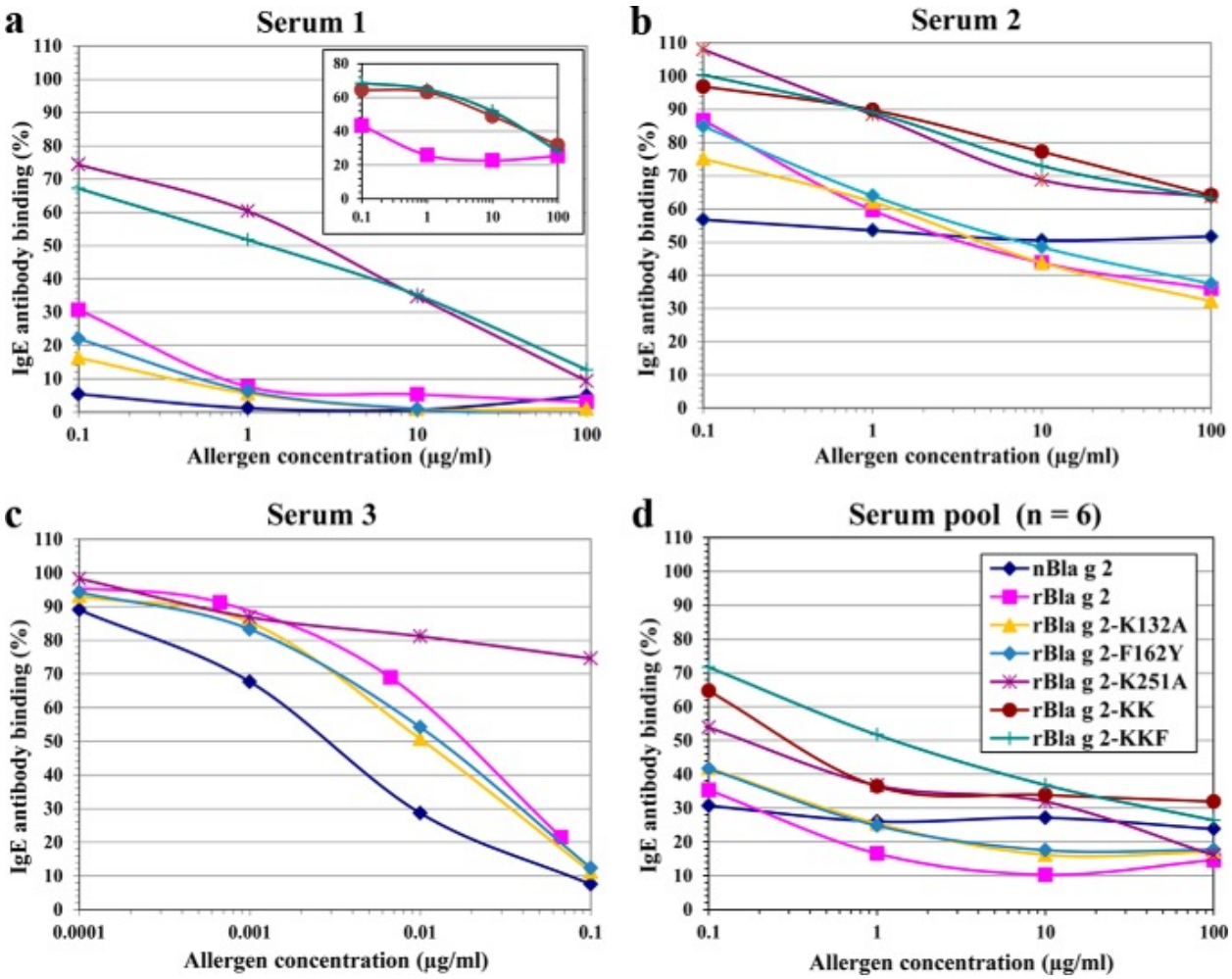

FIGURE 5. Inhibition of IgE antibody binding to rBla g 2 by the mutants. ELISAs were performed using three individual sera (*a–c*) and a sera pool (*n* = 6) (*d*) of Bla g 2-sensitized cockroach-allergic patients. Plots for sera 1 and 3 were very similar for the same mutants and at a range 0.1–100 µg/ml of inhibitor concentrations (shown only for sera 1). Therefore, the plot for serum 3 shows an additional experiment using only the single mutants at a lower inhibitor concentration range (0.0001–0.1 µg/ml). *Inset* in *a* shows inhibition curves from an additional experiment comparing the multiple mutants KK and KKF to rBla g 2.

KKF Mutant Activates Mast Cells. Only a few sera from cockroach-allergic patients had high Bla g 2-specific IgE antibody titers, suitable for mediator release assays. Two out of six sera with high titers of Bla g 2-specific IgE were selected for further analysis of mutants because rBla g 2 induced mediator release similar to positive controls treated with anti-FcϵRI antibody (data not shown). Optimization experiments showed that serum dilution 1:10 was optimal for degranulation when activated with 0.5 µg/ml of nBla g 2. This dilution was therefore used for

testing the ability to induce β-hexosaminidase release by the Bla g 2 mutants. The KKF mutant was analyzed alongside appropriate controls including the following: spontaneous release (6.1 ± 0.0%), anti-FcεRI antibody (14.5 ± 0.3%), nBla g 2 (17.7 ± 9.3%; 21.9 ± 11.5%), and rBla g 2 (ranging from 6.1 ± 1.9 to 15.8 ± 7.5%, depending on the allergen concentrations; average of n = 2 experiments performed in triplicate) (Fig. 6). Compared with recombinant Bla g 2, which induced β-hexosaminidase release similar to the positive controls, no significant difference in release was observed in KKF mutant-activated mast cells (ranging from 5.6 ± 1.2 to 14.6 ± 7.5%). Thus, the KKF mutant is not hypoallergenic in its ability to induce FcεRI-mediated degranulation of human mast cells.

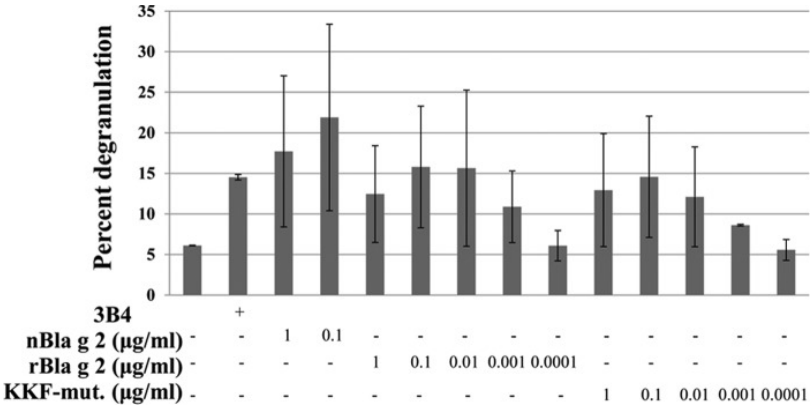

FIGURE 6. KKF mutant does not prevent degranulation from human mast cells compared with rBlag2. Skin mast cells (from five different donors) were incubated with IgE serum (sera G1 or F1; 1:10 dilution) overnight, washed, and cross-linked with or without anti-FcεRI antibody (22E7/1.0 µg/ml), wild type antigen (nBla g 2), recombinant antigen (rBla g 2), or the mutant antigen (KKF) for 30 min. Samples were centrifuged, and β-hexosaminidase release was measured at 405 nm. Results are the average of two separate experiments/donors ± S.D. and are representative of four separate experiments demonstrating similar results. No statistical significant release ($p > 0.05$) was observed between the mutants and rBla g 2.

KKF Mutant Retains the Capacity to Induce T-cell Responses. In PBMC cultures from cockroach-allergic subjects, *B. germanica* extract and Bla g 2 induced significant increases in levels of secreted IL-13 and IL-10 ($p \leq 0.039$), modest increases in IL-5 and IFN-γ, and no change in IL-4 as compared with non-stimulated cultures (Fig. 7). Under non-stimulated conditions, high levels of secreted IFN-γ were observed within the allergic group, whereas IL-10 was the dominant cytokine in the non-allergic group. Thus, although T-cell cytokine profiles in the cockroach allergic group were IFN-γ-dominated in the presence of *B. germanica* extract and Bla g 2, IFN-γ production was only partially attributable to cockroach allergens. The mutant KKF induced comparable $CD4^+$ T-cell reactivity to Bla g 2 as judged by CD25 expression, irrespective of allergic status, indicating that T-cell epitopes were retained (Fig. 8*a*). The KKF mutant induced higher levels of IL-10 as compared with non-stimulated cultures (geometric mean = 1,221 pg/ml (95% CI: 324.1 pg/ml, 4,597 pg/ml) *versus* 83.6 pg/ml (95% CI: 22.2 pg/ml, 314.8 pg/ml) for non-stimulated cells ($p \leq 0.045$)) (Fig. 8*b*). Moreover, KKF induced higher levels of IL-10 as compared with both *B. germanica* extract (geometric mean = 225.6 pg/ml (95% CI: 59.9 pg/ml, 849.6 pg/ml)) ($p < 0.012$) and Bla g 2 (geometric mean = 179 pg/ml (95% CI: 47.5 pg/ml, 674.2 pg/ml)) ($p < 0.004$). Intracellular cytokine staining revealed that activated ($CD25^+$) $CD4^+$ T-cells did not express IL-10 in cultures stimulated with KKF, indicating non-T-cells as the source (Fig. 8*c*). No difference in the induction of the T-helper cytokines IFN-γ, IL-4, or IL-5 was observed for the KKF mutant *versus* Bla g 2; however, in contrast to Bla g 2, this

mutant was a weak inducer of the Th2 cytokine, IL-13 (Fig. 8*b*). These findings, together with altered IgE binding properties, demonstrate the immunomodulatory potential of the KKF mutant.

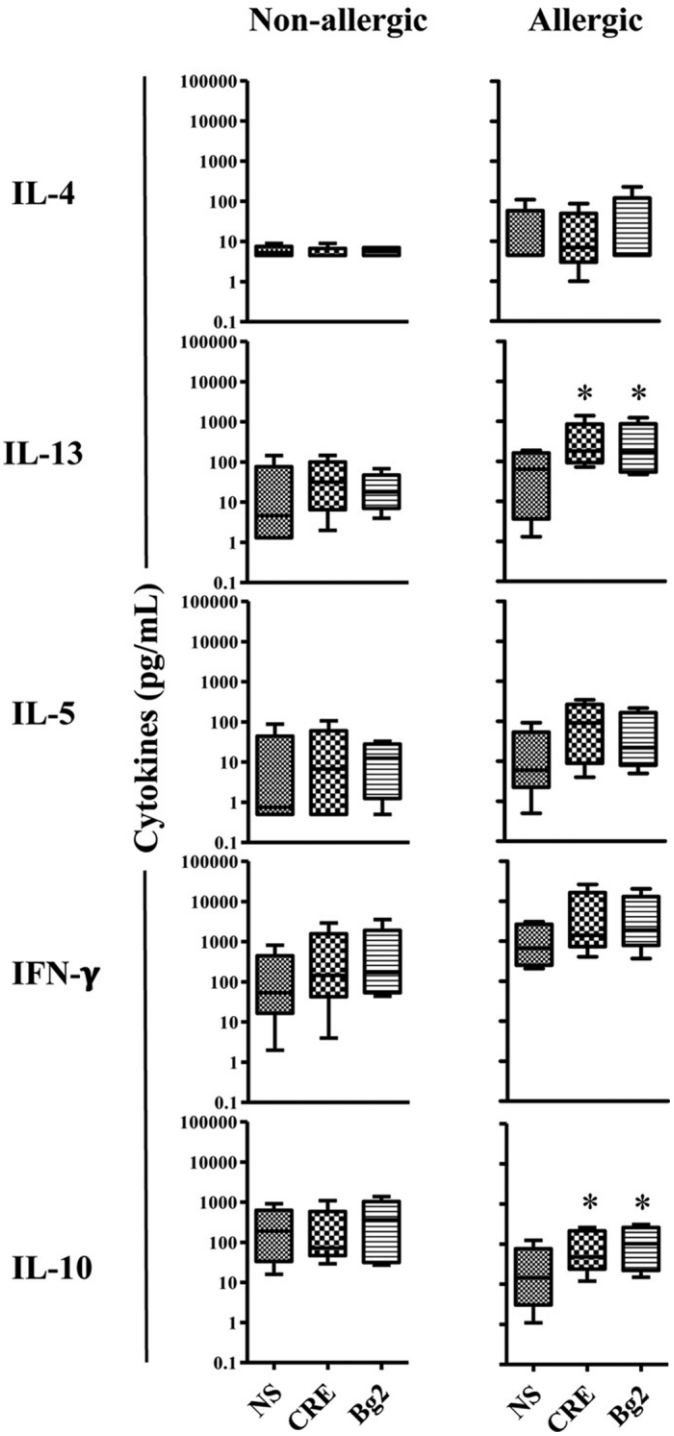

FIGURE 7. Cytokine profiles induced by cockroach allergens. Levels of secreted cytokines were compared between cockroach allergic subjects ($n = 6$) and non-allergic ($n = 4$) subjects in day 7 PBMC cultures (median and interquartile ranges). *NS*, non-stimulated; *CRE*, *B. germanica* extract. *, $p \leq 0.039$ for stimulated *versus* non-stimulated conditions.

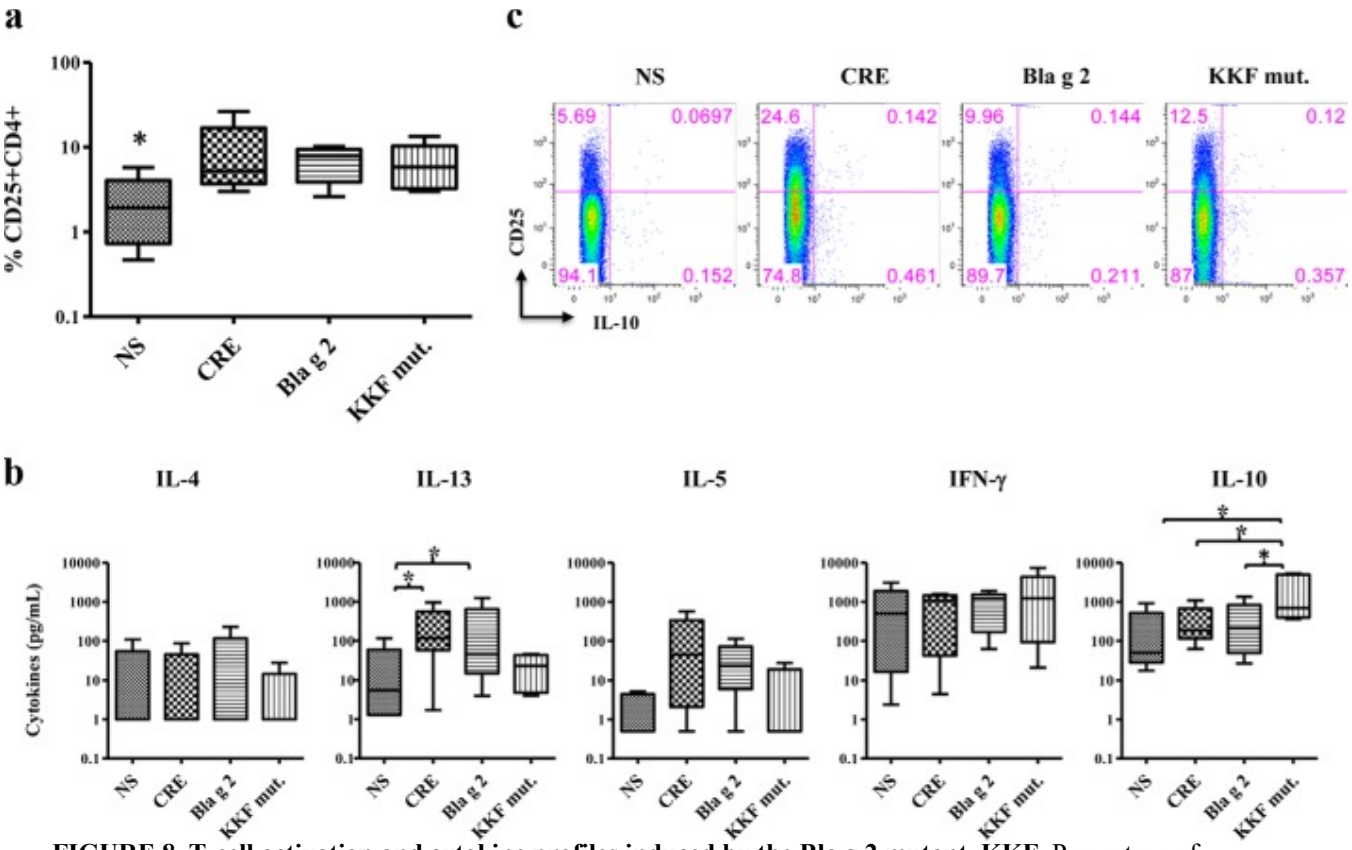

FIGURE 8. T-cell activation and cytokine profiles induced by the Bla g 2 mutant, KKF. Percentage of $CD25^+$ T-cells in CD4 T-cell gate (*a*) and levels of secreted cytokines (*b*) were induced by different stimuli in day 7 PBMC cultures (median and interquartile ranges). Summary data are shown for subjects 1, 2, 4, 7, and 8 in Table 1. *Asterisk* in *a* denotes $p < 0.05$ for non-stimulated *versus* all other stimuli. *, *p* values ≤0.045 in *b. NS,* non-stimulated; *CRE, B. germanica* extract. *c,* gated CD4 T-cells showing expression of surface CD25 against intracellular IL-10 in day 7 PBMC cultures. Data are representative of five subjects.

Most allergens activate $CD4^+$ T-cells via presentation of peptides in the context of HLA-DRB1 molecules. To explore the molecular basis for how point mutations might impact T-cell responses, we first analyzed predicted core epitopes (9-mer peptides) of the KKF mutant as compared with wild type Bla g 2 for 13 HLA-DRB1 supertypes using MULTIPRED2 (28). None of the predicted core epitopes for either the wild type or KKF sequences contained a mutation site (Fig. 9). However, mutation sites were in close proximity to predicted core epitopes, suggesting that they might constitute flanking residues. Using a prediction algorithm that takes into account the contributions of flanking residues to HLA binding (NetMHCIIpan) (29), several 15-mer peptide epitopes were identified that contained mutation sites within both the flanking and core regions (Table 4). Whereas K132A and F162Y mutation effects on predicted HLA binding were limited, the K251A mutation effect was more widespread. Specifically, this residue was predicted to be involved in peptide binding for seven DRB1 molecules (0101, 0401, 0701, 0801, 1201, 1301, and 1401). In addition to modest changes in predicted peptide binding affinity, the introduction of the K251A mutation resulted in the loss of three predicted 15-mer epitopes containing two unique 9-mer core epitopes, along with the creation of 12 new predicted epitopes containing three unique core epitopes. Collectively, these findings predict creation of new T-cell epitopes, without significant loss of wild type epitopes, within the KKF mutant.

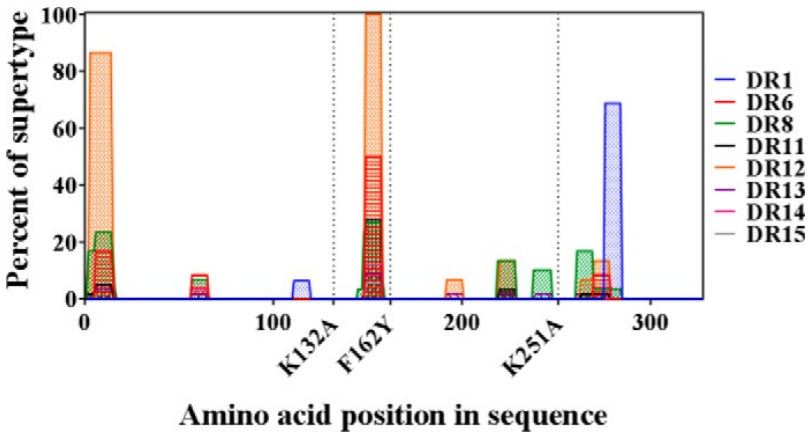

FIGURE 9. KKF mutation sites reside outside predicted core T-cell epitopes. HLA binding for 9-mer peptides of the Bla g 2 KKF mutant were predicted for alleles belonging to 13 HLA-DR supertypes using MULTIPRED2 (28). Results are expressed as the percent of alleles from each super-type that were predicted to bind (IC_{50} of ≤500 nm) according to localization within the primary amino acid sequence. No binding was predicted for DR3, DR4, DR7, DR9, and DR16 supertypes. Location of each mutation site is denoted by a *dotted line*.

Discussion

Antigenic determinants of Bla g 2 involved in IgE antibody recognition were investigated with a view to generating information that could be useful for the design of recombinant mutants as potential candidates for immunotherapy. Most strategies for mapping IgE antibody epitopes of allergens have relied on the screening of peptides, either by immunoblot or microarray, for the identification of linear epitopes (37, 38). Although linear epitopes are common in foods, most IgE epitopes in inhalant allergens are conformational (39, 40). There are no computational tools to accurately predict conformational epitopes, and several prediction methods available identify only linear B-cell epitopes (41,–43). Our approach, which was based on a combination of x-ray crystallography and site-directed mutagenesis, showed the conformational nature of IgE epitopes (13,–16). The strategies for mutagenesis in this study were based on experimentally determined structures of Bla g 2 alone or in complex with antibodies (13,–15). Therefore, the three-dimensional structure of the protein, essential for induction of immune responses by inhaled allergens, was an important consideration in our study.

Interestingly, a comparative structural analysis of Bla g 2 alone or in complex with mAb 7C11 or 4C3, revealed a mechanism of antibody recognition, by which each of the two lysines 132 and 251 underwent a conformational change upon antibody binding. During initial antibody binding, the negatively charged aromatic ring of a tyrosine in the antibody attracts a positively charged lysine in the allergen, resulting in formation of a cation-π interaction. Cation-π interactions (which we previously reported for Lys-132 and Lys-251) involve non-covalent binding between a cationic group (Arg and Lys) and an electron-rich aromatic residue (Trp, Phe, and Tyr) (14,–16, 44). This attraction contributes to orienting the antibody in a manner that facilitates a close interaction with Bla g 2 and resultant hydrogen bond formation. Thus, our findings highlighted the importance of the cation-π interactions in the initial stages of allergen-antibody recognition, *i.e.* before formation of the definitive hydrogen bonds.

TABLE 4, Comparison of predicted HLA-DR binding for 15-mer peptides of wild type Bla g 2 and its KKF mutant. Peptide binding predictions were performed for 12 alleles using NetMHCIIpan (29). Peptides with binding affinities of IC50 ≤ 500 nm were considered to be predicted binders. Red amino acids indicate the position of the KKF mutation sites within the peptide sequences. n.b. no binding.

	Allele (HLA-DRB1)	15mer Epitope	WT Core	WT IC_{50} (nM)	KKF Core	KKF IC_{50} (nM)
K132A	0101	VVVGIAAPGCPNALA	VVGIAAPGC	416.92	VVGIAAPGC	412.58
	0101	APGCPNALAGKTVLE	PNALKGKTV	490.31	PNALAGKTV	460.28
	0101	PGCPNALAGKTVLEN	PNALKGKTV	423.5	PNALAGKTV	417.43
	0101	GCPNALAGKTVLENF	PNALKGKTV	350.61	PNALAGKTV	364.4
	0101	CPNALAGKTVLENFV	PNALKGKTV	334.58	PNALAGKTV	386.56
F162Y	0801	VFSIHHARFQDGEHY	VFSIHHARF	424.97	VFSIHHARF	432.54
	1201			55.74		56.99
	1201	FSIHHARFQDGEHYG	SIHHARFQD	435.28	SIHHARFQD	470.82
	1201	HYGEIIFGGSDWKYV	IIFGGSDWK	430.53	IIFGGSDWK	434.31
K251A	1201	EKTTTRRICKLDCSA	TRRICKLDC	307.42	TRRICKLDC	334.9
	0801	KTTTRRICKLDCSAI	ICKLDCSKI	380.04	ICKLDCSAI	412.12
	1201			129.44		178.05
	0101	TTTRRICKLDCSAIP	ICKLDCSKI	n.b.	ICKLDCSAI	375.1
	0801			429.72		456.09
	1201			121.69		180.11
	1301			n.b.		473.51
	0101	TTRRICKLDCSAIPS	ICKLDCSKI	491.04	ICKLDCSAI	239.82
	0801			432.15		475.05
	1201			117.67		191.92
	1301			n.b.		369.04
	0101	TRRICKLDCSAIPSL	ICKLDCSKI	281.51	ICKLDCSAI	139.29
	0401			n.b.	CKLDSCAIP	339.05
	0701			432.66	ICKLDCSAI	355.55
	0801			379.35		444.51
	1201			91.46		161.7
	1301			340.85		243.33
	1401			492.14		n.b.
	0101	RRICKLDCSAIPSLP	ICKLDCSKI	292.86	ICKLDCSAI	152.77
	0401			n.b.	CKLDSCAIP	369.55
	0701			429.8	ICKLDCSAI	358.05
	0801			439.04		n.b.
	1201			107.35		200.27
	1301			336.98		255.39
	0101	RICKLDCSAIPSLPD	ICKLDCSKI	440.72	ICKLDCSAI	215.31
	0401			n.b.	CKLDSCAIP	463.62
	0701			n.b.	ICKLDCSAI	436.17
	1201			168.66		300.99
	1301			444.8		355.8
	0101	ICKLDCSAIPSLPDV	ICKLDCSKI	n.b.	CSAIPSLPD	248.84
	0701			n.b.		468.05
	1201			414.43		n.b.
	0101	CKLDCSAIPSLPDVT	None	n.b.	CSAIPSLPD	300.53
	0101	KLDCSAIPSLPDVTF	None	n.b.	CSAIPSLPD	299.82
	0101	LDCSAIPSLPDVTFV	None	n.b.	CSAIPSLPD	448.94

The main goal was to express a Bla g 2 multiple mutant with substitutions of important residues for IgE antibody binding to different epitopes that would display a reduced IgE antibody binding capacity, without loss of the overall fold, while keeping the capacity to modulate T-cell responses. This approach, by retaining the overall molecular structure of the allergen, should preserve its capacity to induce IgG antibodies with similar specificities to the wild type (given that only few residues would be modified), which would be capable of interfering with allergen-IgE interactions during immunotherapy (45). Three residues located in three separated areas on the surface of Bla g 2 were selected. Two were lysines 132 and 251 that had previously been shown to be key residues for IgE antibody binding by analysis of allergen-antibody complexes (14,–16). The third was the aromatic residue phenylalanine 162, which was newly identified as an IgE antibody-binding site. Also, in search of putative additional IgE antibody-binding sites, the importance of the molecular quasi-symmetry of Bla g 2 for antigenicity was investigated by a comparison with truly symmetric structures of homologous retroviral enzymes. This third approach was newly designed, and it embraces a few of the criteria used by bioinformatic tools for prediction of B-cell epitopes (surface accessibility, protruding areas, or geometrical shape), and thus was novel. No dominant IgE epitope was found in Bla g 2 in a symmetrical position opposite to a known IgE antibody-binding site in Bla g 2. Overall, these results indicate that a combination of the identity and position of an amino acid on the molecular surface of Bla g 2, rather than only either identity (*i.e.* Phe) or position (*i.e.* symmetric location in protruding opposite lobes) of the residue, determines IgE antibody epitopes in Bla g 2.

The most remarkable finding is that only 2–3 amino acid substitutions (in KK and KKF mutants), each in a different epitope on the surface of Bla g 2, led to a significant reduction of IgE antibody binding (∼100-fold). This result points toward a low number of relevant IgE antibody-binding sites for Bla g 2, suggesting that IgE responses are oligoclonal in nature, as shown for other allergens (46,–48). A single mutation in an epitope, as performed here for three epitopes, is expected to lead to a reduction of antibody binding affinity, which may or may not affect mediator release, depending on the IgE clonality and the affinity of the remaining epitopes for IgE (49). Not surprisingly, the triple mutant KKF was still able to induce β-hexosaminidase release from mast cells. Future studies modifying more than one residue per IgE epitope and/or additional epitopes will most likely lead to the production of hypoallergens. As desired, the combination of three mutations did not disrupt the overall fold of the allergen, proven by the x-ray crystal structure of the triple mutant that perfectly overlapped with the structure of rBla g 2-N93Q. Preservation of the overall molecular structure pointed to local changes introduced by the mutations, as opposed to overall molecular misfolding. Many approaches to improve allergy vaccines depend on reduction or elimination of IgE antibody binding by disrupting the tertiary structure of the allergen, to avoid anaphylactic effects during vaccination. Conformational variants of allergens that display reduced IgE binding have been engineered using a variety of methods, including disruption of disulfide bonds, proline substitutions, and generation of fragment hybrids (50,–55). In relation to this point, a misfolded Bla g 2 mutant displayed reduced capacity to bind IgE and immunomodulatory potential (data not shown). However, the goal of this study was to modify IgE antibody-binding epitopes without loss of the overall three-dimensional structure to preserve the capacity to induce IgG for immunotherapy purposes. The Bla g 2 mutant KKF retained the capacity to induce T-cell reactivity and modulated cytokine profiles in *in vitro* cultures. Consistent with these observations, T-cell epitope prediction algorithms indicated changes in T-cell epitopes within the KKF mutant that would not be

expected to reduce $CD4^+$ T-cell reactivity as compared with wild type allergen. T-cell responses to Bla g 2 among cockroach allergic subjects were distinguished from those in non-allergic subjects by the induction of higher levels of IL-13 and IL-10. Interestingly, the prototypic Th2 cytokine, IL-4, was not a feature of the cytokine response in allergic subjects, as measured by both secretion and intracellular expression (data not shown). Despite this, our ability to detect IL-13 in the presence of Bla g 2 supports its competence to induce atypical IL-13+ Th2 effectors. This observation deviates from work reporting that responses induced by Bla g 2 epitopes are IFN-γ-restricted (18). However, in those studies, IL-13, which was the most abundant Th2 cytokine in our system, was not assessed. The mutant KKF induced higher levels of the suppressive cytokine IL-10 compared with Bla g 2, and it was a weak inducer of IL-13 compared with Bla g 2. Importantly, the mutant KKF displayed T-cell modulatory properties while retaining the native allergen fold. The rarity of IL-10-expressing $CD4^+$ T-cells in cultures stimulated with KKF suggests that the principal source of IL-10 is antigen-presenting cells as opposed to IL-10-expressing peripheral regulatory T-cells. It is tempting to speculate that subtle molecular changes in Bla g 2 dampen Th2 priming through induction of IL-10 by dendritic cells. This could occur through reduced binding to the high affinity IgE receptor (FcεRI) by virtue of diminished interactions with IgE or else through enhanced binding to pattern recognition receptors via the creation of new molecular motifs (56). The capacity for Bla g 2 mutants to impact antigen-presenting cells has important consequences for immune networks beyond modulating T-cell responses, and this aspect warrants further study.

Studies carried out over the past 20 years by the National Institutes of Health Inner-City Asthma Consortium (ICAC) have highlighted the importance of cockroach allergy as a cause of asthma morbidity in the United States (5, 6). The development of structurally engineered recombinant allergens, designed as described here, could provide the next generation of allergen vaccine candidates. Taken together, our findings show that it is possible to generate Bla g 2 mutants with reduced capacity to bind IgE that preserve a well defined three-dimensional structure and the capacity to modulate T-cell responses. The advent of recombinant IgE antibody technologies combined with structural analysis of allergen-antibody complexes should allow the precise identification of IgE antibody-binding sites for the design of attractive candidates for a cockroach allergy vaccine. Such a vaccine might consist of a combination of Bla g 2 hypoallergens with additional cockroach allergens tailored to the unique IgE sensitivity profile of each cockroach-allergic patient.

Author Contributions

J. G. was responsible for allergen mutagenesis, expression, purification, and immunological testing, under the supervision of A. P. P. W. W. performed the T-cell experiments under the supervision of J. A. W. C. L. K. performed mast cell release assays. Structure determination and structural analysis were performed by M. L., A. G., and A. W. A. G. interpreted structures and prepared the figures related to structure determination. M. H. collected the CD spectra and performed mass spectrometry analysis of mutants. L. M. M. performed all *in silico* analyses of T-cell epitopes and assisted with manuscript preparation. A. P. conceived and coordinated the study and wrote the paper, which was critically revised for intellectual content by J. A. W., A. W., A. G., and M. D. C. All the authors approved the final version of the manuscript.

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The atomic coordinates and structure factors (code 4RLD) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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